Modulation of the Polymorphism of the Palmitic Acid/ Cholesterol System by the pH

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Differential scanning calorimetry, infrared spectroscopy, solid-state ^{13}C and ^{2}H NMR spectroscopy, and X-ray diffraction were used to characterize hydrated mixtures of palmitic acid (PA) and cholesterol (chol), at low (5.4) and high (8.5−9.0) pH. The pH affects the ionization state of the fatty acid, and it was found to modulate its interaction with cholesterol, and the architecture of the resulting complexes. At low pH, this system undergoes a transition from a phase-separated crystalline state to a lamellar liquid-ordered (lo) phase, in agreement with previous results (*Langmuir* **2001**, *17*, 5587−5594). The apparent pK_a of PA in these conditions is found to be about 8.2. At high pH, when the fatty acid is deprotonated and consequently negatively charged, both components participate in the formation of an lo lamellar structure that was found to be stable over a large temperature range (20−70 °C). For 50/50 PA/chol mixtures, only this phase, with a *d* spacing of approximately 49 Å, is detected. These fluid bilayers can be characterized as having solubilized cholesterol and highly ordered PA hydrocarbon chains. It is thus proposed that the electrostatic repulsion between carboxylate headgroups allows cholesterol molecules to integrate into the palmitate matrix. The sterol promotes the bilayer formation and leads to an ordering of the PA acyl chain, a well-known effect of cholesterol with phospholipids. Therefore, the PA/chol system allows for the production of very simple lo lamellar structures that are pH-sensitive.

Introduction

It is now well-known that the presence of cholesterol (chol) in phospholipids membranes strongly influences the behavior of these membranes. One of the most remarkable features is the formation of the liquid ordered (lo) phase, a phase that shares characteristics with both the gel and the liquid crystalline (also referred to as the liquid disordered) phases. For the lo phase, the rotational and translational lipid mobilities are similar to those of the liquid disordered phase, while, from an intramolecular point of view, the phospholipid chains are highly ordered.[1−5] The formation of lo phases in cholesterol-containing phospholipid bilayers has been previously reported for phospholipids with various headgroups and acyl chains,[1−3,5,6] suggesting the ubiquitous presence of lo phases in phospholipid membranes. For simpler lipids such as fatty acids, the influence of cholesterol has been less extensively studied. Langmuir−Blodgett monolayer studies have concluded that long saturated fatty acids and cholesterol are immiscible, or slightly miscible, in the condensed state,[7−9] whereas, in the expanded state, they are found to be totally miscible.[10] Recently, a composition−temperature diagram was proposed for the palmitic acid (PA)/chol system at pH 5.5.[11] An interesting feature of this diagram is that, despite the immiscibility of the components at low temperatures, the formation of a lamellar lo phase was reported for temperatures higher than 50 °C. This finding shows the prominent ability of cholesterol to induce lo lamellar phases and has resulted in efforts to examine the minimal molecular requirements to form lo phases. Since the pH dictates the protonation state of the polar headgroup of fatty acids, we have examined the influence of this physical parameter on the formation of lamellar lo phases using the PA/chol system. In addition to this fundamental aspect, the effect of pH on the fatty acid−cholesterol system is of special interest for the lipids in the stratum corneum. Fatty acids and cholesterol are, as ceramides, the main lipid components in the stratum corneum (SC), the top layer of the skin. The polymorphism of the SC lipids plays a key role in skin properties, as illustrated by its intimate link with skin permeability.[12,13] There is a pH gradient found across the skin, which ranges from pH 7.4 to about pH 5 at the skin surface[14,15] and whose potential influence on SC lipid polymorphism remains to yet be defined. By examining the pH influence on PA/chol systems, the present work provides constructive knowledge in attempting to gain a better understanding of this aspect.

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Ionized, saturated fatty acids in water give rise to a rich and complex polymorphism that is dependent on several factors, including the nature of the counterion, the amount of water, temperature, the concentration of the amphiphile, and the ionic strength of the aqueous phase.[16] In general, in aqueous media the deprotonated fatty acids undergo, upon heating, a transition from a crystalline phase to micelles[17] (the solubility of deprotonated fatty acids in water is still limited; e.g. the solubility of potassium laurate was estimated to 0.02 M[18]). The effect of cholesterol on ionized fatty acids has received little attention, but some reports show that these mixtures display complex phase behaviors. For example, a matrix of hydrated and semiionized palmitic and oleic acids with cholesterol forms two different crystalline phases as well as one gel phase.[19] The present work investigates the effect of pH and temperature on the phase behavior of hydrated mixtures of palmitic acid and cholesterol. We have characterized the PA/chol mixtures at pH values between 5.5 and ~8.7 using ^{13}C- and ^{2}H-nuclear magnetic resonance (NMR) spectroscopy, infrared (IR) spectroscopy, differential scanning calorimetry (DSC), and X-ray diffraction (XRD). These complementary techniques have provided us with both molecular and nanoscopic details of the resulting structures. Moreover, this work reports additional information regarding the behavior of the PA/chol mixtures at low pH and examines the phase behavior of the palmitate/chol mixtures at high pH, to highlight the impact of a negative carboxylate group on the structures adopted by this simple (pseudo)binary system.

Materials and Methods

Material. Palmitic acid (approximately 99%), cholesterol (>99%), and MES (2-[*N*-morpholino]ethanesulfonic acid) (minimum 99.5%) were purchased from Sigma Chemical Co. (St. Louis, MO) and used without further purification. Deuterated palmitic acid (PA-d_{31}) was obtained by deuterating palmitic acid (Sigma), using the method previously described.[20] Sodium borate (laboratory grade) and sodium chloride (≥99.0%) were obtained from Anachemia (Montréal, Canada).

Samples Preparation. The mixtures were prepared by dissolving weighted amounts of palmitic acid and cholesterol in a mixture of benzene/methanol (95/5 (v/v)). The solutions were then frozen in liquid nitrogen and lyophilized for at least 15 h to allow the complete sublimation of the organic solvent.

For the DSC studies, the lipid powder was hydrated with MES buffer (5 mM MES, 5 mM NaCl, and 0.5 mM EDTA) in the case of the low-pH samples and with borate buffer (5 mM sodium borate, 5 mM NaCl, and 0.5 mM EDTA) for the high-pH samples. The samples were submitted to at least three freeze-and-thaw cycles, from liquid nitrogen temperature to 65 °C, with frequent vortexing. The pH was then measured and readjusted, if necessary, with a dilute solution of HCl or NaOH. After each pH adjustment, the freeze-and-thaw cycle was repeated and the samples were incubated for at least 1 day before remeasuring the pH. This procedure was necessary especially for the high-pH samples, because pH equilibration often took several hours, a phenomenon also noted by Small.[16] The final overall concentration of the suspensions was about 21 mg of total lipids/(mL of buffer).

For IR spectroscopy, the sample preparation was similar except that a MES buffer (100 mM, 25 mM NaCl, and 5 mM EDTA) was used for pH 6.5 or less, a Hepes buffer (100 mM, 25 mM NaCl, and 5 mM EDTA), between pH 7 and 8.5, and a borate buffer (100 mM, 25 mM NaCl, and 5 mM EDTA) for pH 9 and higher. The buffers were prepared in D_2O in order to avoid the spectral interference of the H_2O deformation band with the C−O stretching mode of the acid. The final overall lipid concentration was 150 mg of total lipids/(mL of buffer).

For the ^{2}H NMR spectroscopy, a similar procedure was followed except that the buffer was a 20 mM Hepes, containing 100 mM NaCl and 2 mM EDTA, prepared in deuterium-depleted water. The final overall lipid concentration was 225 mg of total lipids/(mL of buffer).

For ^{13}C NMR and X-ray diffraction studies, the preparation was similar to the DSC samples, except that the initial concentration of the suspensions was 40 mg of total lipids/(mL of buffer) (5 mM MES or sodium borate, 5 mM NaCl, and 0.5 mM EDTA). When the pH was stabilized, the aqueous suspension was lyophilized. The dry powder was then hydrated with pure water to a proportion of about 500 mg of total lipids/mL. This modified procedure led to a final buffer composition of 30 mM MES or sodium borate, 30 mM NaCl, and 3 mM EDTA. This approach was found to provide more accurate pH measurements as it is rather difficult to perform this operation on concentrated lipid suspensions.

Differential Scanning Calorimetry. The differential scanning calorimetry was performed with a VP-DSC microcalorimeter (MicroCal, Northampton, MA). An aliquot of 0.4 mL of the suspension was transferred in the 0.5 mL sample cell. We did not fill the cell, ensuring a proper detection of all the Cp variations in the samples, even in those showing macroscopic phase separations. The reference cell was filled with deionized water. The heating and cooling rates were typically 15 °C/h. Data acquisition and treatment were done with the Origin software (Microcal software, Northampton, MA).

IR Spectroscopy. An aliquot of the sample was placed between two CaF_2 windows separated by a 5 μm thick Teflon ring. This assembly was put in a brass sample holder whose temperature was controlled, using Peltier thermopumps. The IR spectra were recorded on a BioRad FTS-25 spectrometer, equipped with a water-cooled globar source, a KBr beam splitter, and a midrange mercury−cadmium telluride detector. Each spectrum is the result of 100 scans with a 2 cm^{-1} resolution. The temperature was varied from low to high temperature with a 12 min period introduced to allow for temperature equilibration prior to data acquisition.

^{2}H NMR Spectroscopy. The spectra were recorded on a Bruker DSX-300 spectrometer, using a Bruker static probe equipped with a 5 mm coil. A quadrupolar echo sequence was used with a 90° pulse of 4.2 μs and an interpulse delay of 35 μs. The recycling time was 500 ms (the absence of slow-relaxation component was verified using a 50 s recycling delay). After the second pulse, 8192 points were recorded in quadrature mode, with a dwell time of 0.5 μs. Typically 20 000 FID were co-added. The temperature was regulated using a Bruker VT-100 controller.

^{13}C NMR Spectroscopy. The spectra were also recorded on a Bruker DSX-300 spectrometer, operating at 75 MHz for ^{13}C. A 4 mm Bruker MAS probe was used. The cross-polarization time was typically 1 ms, and the recycling time was 7 s. The sample was rotated at the magic angle with a frequency of 5000 Hz. The temperature was monitored by a Eurotherm temperature controller. The chemical shifts are referred to with respect to the signal of the carbonyl ^{13}C of glycine (external standard) at 176.03 ppm.

X-ray Diffraction. Powder XRD data were obtained at CHESS (Ithaca, NY) using the D-1 station. Monochromatic X-rays with $\lambda = 1.03$ Å were obtained using a tungsten/silicon multilayer having a repeat spacing of 22.5 Å and a mosaic of ~0.25°. The diffraction patterns were detected using a two-dimensional charged coupled device (CCD) detector with a 1024 × 1024 array of pixels, each of active area of 47 μm × 47 μm. The distance from the sample to the CCD detector was 0.262 m.

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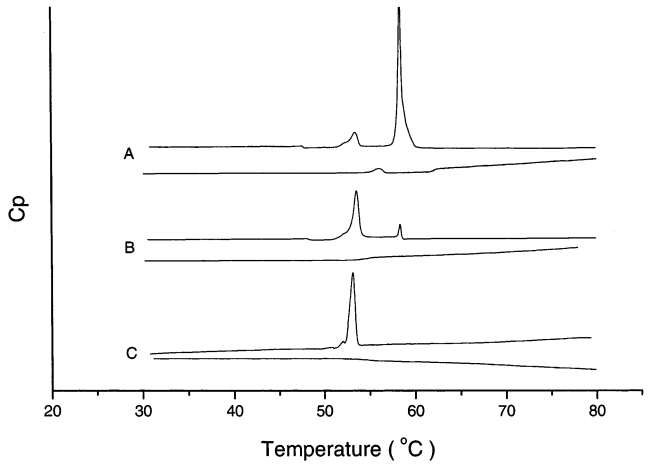

Figure 1. DSC thermograms of PA/chol mixtures: (A) 75/25 PA/chol, pH 5.4 (top) and 9.0 (bottom); (B) 50/50 PA/chol, pH 5.4 (top) and 9.0 (bottom); (C) 25/75 PA/chol, pH 5.4 (top) and 9.0 (bottom). The heating rates were 15 °C/h.

Results

Differential Scanning Microcalorimetry. Figure 1 shows the thermograms of various PA/chol mixtures, at pH 5.4 and 9. At pH 5.4, where PA can be considered as fully protonated (see ref 11, and results below), the samples appeared as suspensions of visible solid particles in a clear liquid phase. Two endothermic peaks were detected for the samples with a PA/chol molar ratio of 75/ 25, and 50/50, at pH 5.4. The first one, at 53.5 °C, has been attributed to the formation of a liquid-ordered phase,[11] whereas the second endothermic peak, appearing at 58.5 °C, was attributed to the melting of almost pure palmitic acid domains. For comparison, the melting of pure palmitic acid in an excess of MES buffer was identified by DSC at 61.5 °C (data not shown). The temperatures associated with the maxima of both peaks remained constant for the different amounts of cholesterol present in the samples, suggesting that the variable cholesterol content changed the proportion of two phases but not their composition. When the cholesterol proportion reached 75 mol %, only the peak at 53.5 °C was observable. Under these conditions, the presence of a eutectic behavior has been proposed,[11,22] in agreement with the calorimetric data.

Crystalline monohydrated cholesterol undergoes a dehydration transition around 72 °C, a transition that we observed for cholesterol in excess water as in other studies.[23,24] In the thermograms of the PA/chol mixtures, no transition over 70 °C was observed, suggesting that cholesterol was not in this crystalline phase but was most likely completely solubilized in the palmitic acid matrix, for proportion ≤75 mol % chol. In addition, we have never observed, in these mixtures, the polymorphic transition of anhydrous cholesterol, from the anhydrous I to the anhydrous II crystalline form, that occurs at approximately 33 °C.[24] This transition is observed even when dehydrated crystalline cholesterol is in water (data not shown). Therefore, this result suggests that cholesterol recrystallizes to the monohydrate form in the presence of palmitic acid, even if the mixture has been heated over the normal dehydration temperature of this sterol. This comportment is different from the one observed with pure cholesterol in excess water, where the complete rehydration process is very slow, requiring at least 18 h at 20 °C.[24]

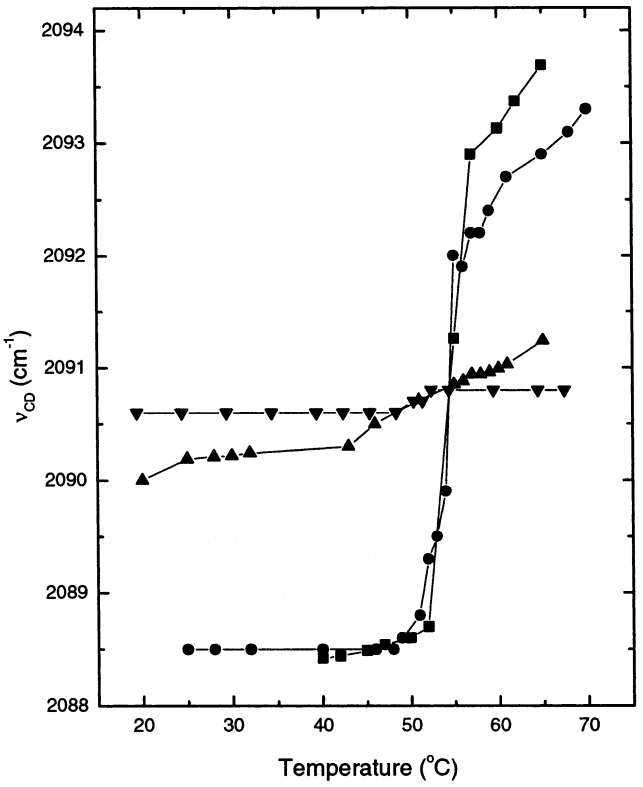

Figure 2. Thermotropism of a 50/50 PA-d_{31}/chol mixture probed by the position of the ν_{C-D} band for pH 5.5 (■), pH 7.5 (●), pH 8.4 (▲), and pH 8.7 (▼).

When the pH was raised to 9, the thermotropism of the PA/chol mixtures was different. Visually, the mixtures generally appeared as homogeneous opalescent dispersions similar to standard phospholipid suspensions. Moreover, the thermograms did not exhibit the transitions observed for the low-pH samples. For the 75/25 PA/chol mixtures, only a very small transition was observed at 56 °C. It has been shown that the melting transition for hydrated salts of fatty acids generally occurs a few degrees below the melting temperature of the analogous protonated fatty acids.[25,26] In our case, the small transition occurred about 5 °C below the melting point of hydrated palmitic acid (61.5 °C), suggesting that it may be attributable to the presence of acid-soap crystals. This explanation is also consistent with the presence of small amounts of precipitate in these samples. For the 50/50 and 25/75 PA/chol mixtures, no transition was detected between 30 and 80 °C. It must be highlighted that the cholesterol dehydration transition was also absent from the thermograms and concluded that both PA and cholesterol participate in the formation of a stable structure.

IR Spectroscopy. Equimolar PA-d_{31}/chol mixtures at different pH were investigated using IR spectroscopy. Deuterated acid was used to eliminate the spectral interference of cholesterol with the palmitic acid bands. Figure 2 shows the variation of the position of the C−D symmetric stretching (ν_{CD}) vibration of the deuterated methylene groups, observed at about 2090 cm^{-1}. This mode is known to be mainly sensitive to the trans-gauche

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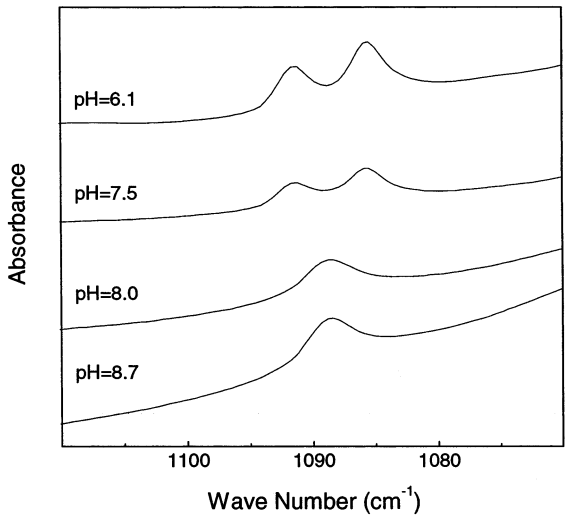

Figure 3. pH dependence of the CD_2 deformation band shape, for 50/50 PA-d_{31}/chol mixture (25 °C).

isomerization and is commonly used to probe phase transitions.[27] Between pH 5.5 and 7.5, the ν_{CD} mode appeared at 2088.5 cm^{-1}, a value generally observed for highly ordered chains,[11,28] such as in crystalline material. However, between 50 and 55 °C, there was an abrupt increase in the frequency of ν_{CD} to reach values slightly higher than 2093 cm^{-1}, values typical for disordered chains.[27] This variation is therefore characteristic of a crystalline-to-disordered phase transition. At pH 8.4 and 8.7, the abrupt increase in ν_{CD} frequency was no longer observed. In contrast, the band position was observed at about 2090 cm^{-1} and remained relatively constant over the investigated temperature range. This value has already been observed in deuterated phospholipids forming an lo phase in the presence of cholesterol.[3,28,29]

The variation of chain order as a function of pH was also examined for the equimolar PA-d_{31}/chol mixture, using the CD_2 deformation mode, at 1090 cm^{-1} (Figure 3). At low pH, this mode split into two components located at 1091.5 and 1086 cm^{-1}, indicating the presence of crystalline fatty acid chains packed in an orthorhombic symmetry.[30] This splitting results from an interchain coupling that is only possible when the vibrations of adjacent molecules have similar frequencies[31] and also implies that cholesterol was practically excluded from these domains, as the splitting was maximal.[30−32] At pH 8 and above, the splitting was no longer observed with the CD_2 deformation mode that gave rise to a single component at 1088 cm^{-1}.

The state of the protonation of the carboxylic group has been examined by IR spectroscopy. This technique is very appropriate in addressing this issue because the protonated and unprotonated forms give rise to two well-resolved bands. The C═O stretching of the carboxylic group and the CO stretching of the carboxylate group are observed at about 1700 and 1550 cm^{-1}, respectively. Figure 4 displays this region of the IR spectra for equimolar PA-d_{31}/chol mixtures at different pH. At low pH (6.5 and

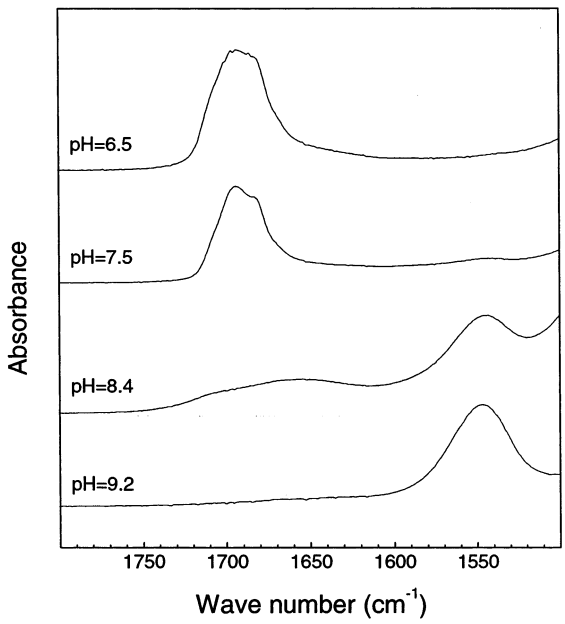

Figure 4. pH dependence of the ν_{CO} mode position of the palmitic acid carboxylic group in 50/50 PA-d_{31}/chol mixture (25 °C).

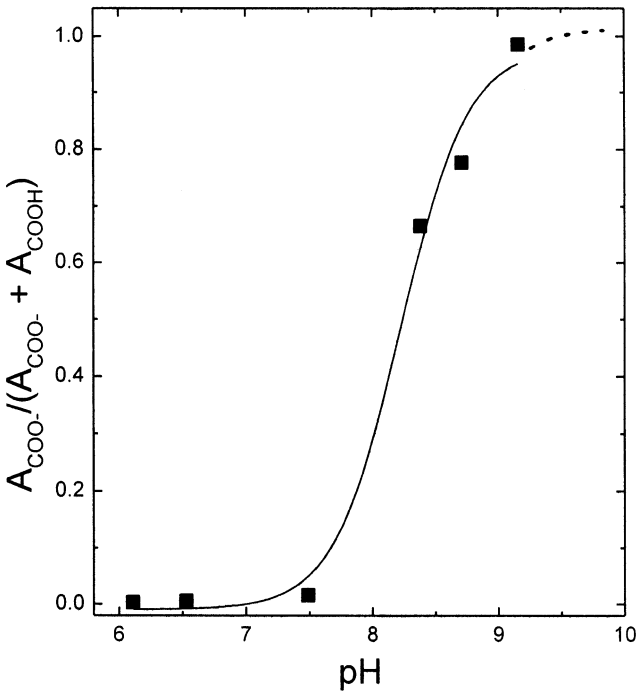

Figure 5. IR titration of the palmitic acid in the 50/50 PA-d_{31}/chol mixture (25 °C).

below), the C═O stretching of the carboxylic group was observed at 1700 cm^{-1}, whereas no contribution was detected at 1550 cm^{-1}, indicating that, in these conditions, the acid was completely protonated. For pH 9.2 and higher, only the band at 1550 cm^{-1} was observed, indicating the fully unprotonated state of the acid. For intermediate pH, the above-mentioned bands were both visible in the spectra. Titration of the acid, as followed by IR spectroscopy, is presented in Figure 5. The area of the bands assigned to the carboxylate (integrated between 1513 and 1602 cm^{-1}) and carboxylic group (integrated between 1610 and 1735 cm^{-1}) vibrations were normalized using the area of the CD stretching mode (integrated between 2050 and 2245 cm^{-1}), to take into account the differences in

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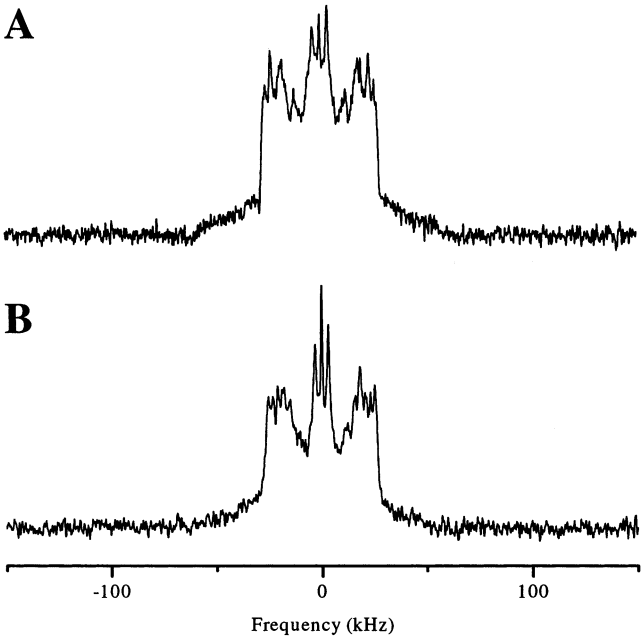

Figure 6. ^{2}H NMR spectra of 50/50 PA-d_{31}/chol mixture at pH 8.8, at (A) 30 and (B) 60 °C.

integrated absorption coefficients of the protonated and unprotonated acid. On the basis of these results, the pK_a of equimolar PA-d_{31} in equimolar proportion with cholesterol, at 25 °C, is estimated to be 8.2.

^{2}H Nuclear Magnetic Resonance Spectroscopy. To provide a detailed description of the acyl chain order of the fatty acid in PA/chol mixtures at high pH, ^{2}H NMR spectra were collected from samples prepared with deuterated fatty acid. Figure 6 shows typical spectra obtained for 50/50 PA-d_{31}/chol mixtures, at pH 8.8—the ^{2}H NMR spectra of the mixtures at pH 5.5 have been reported elsewhere.[11] These spectra, composed of several overlapping powder patterns associated with systems with axial symmetry, are typical of perdeuterated chains in fluid bilayers.[33,34] The quadrupolar splitting measured for the outermost doublet was about 53 kHz, a value that is similar to those previously reported for cholesterol-containing bilayers formed by phospholipids[2,3,34] as well as by PA/chol mixtures at low pH and temperatures above 53 °C.[11] This spectral signature was present over all of the investigated temperature range (30−60 °C). The smoothed orientational order profiles were extracted from these spectra using a standard procedure.[11,21] It is known that the order parameter (S_{C-D}) of carbon 2 of the acyl chain of PA-d_{31} (carbon 1 being the carboxylic carbon atom) exhibits a distinctly small value due to the different orientation of the C2−D bond relative to the normal of the bilayer.[35] It was assumed, mainly to facilitate the comparison between profiles, that this behavior was maintained for the palmitate. Thus, the value of $S_{C-D}(n=2)$ was based on previous assignments.[35] As seen in Figure 7, the order profile is typical of a bilayer, showing a plateau near the headgroup region where the order parameters (S_{C-D}) do not vary considerably as a function of carbon position, followed by a rapid decrease of the orientational order parameter toward the end of the chain. The S_{C-D} values near the headgroup were about 0.43. These values

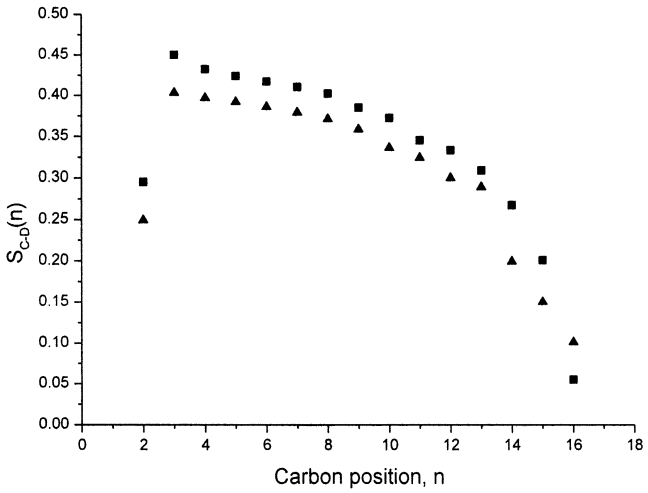

Figure 7. Orientational order profile for the 50/50 PA-d_{31}/chol mixture at pH 8.8 and $T = 60$ °C (■). For comparison purposes, the order profile for the same system at pH 5.5 and $T = 55$ °C (▲) is shown (reprinted with permission from ref 11. Copyright 2001 American Chemical Society).

are typical for lipids in the liquid-ordered phase.[2,3,34] For comparison, the orientational order profile of the equimolar PA-d_{31}/chol mixture at pH 5.5[11] is also shown for a similar temperature.

^{13}C Nuclear Magnetic Resonance Spectroscopy. So far, most of the resulting information is related to the fatty acid component in the mixtures. To examine the behavior of cholesterol, ^{13}C CP-MAS NMR spectroscopy was performed. Figure 8 displays ^{13}C CP-MAS NMR spectra of palmitic acid and cholesterol as individual components, at pH 5.4. The partial peak attribution for palmitic acid was made on the basis of that reported for the spectra obtained in solution (Sadtler ^{13}C NMR spectra library), whereas that of cholesterol was based on previous work.[36] At pH 5.4, the spectrum of the hydrated solid acid recorded at 25 °C (Figure 8A) showed a signal at 181 ppm, attributed to the carbon of the carboxylic group, while peaks in the region between 10 and 40 ppm are attributed to the carbons of the acyl chain. The signal associated with C16 was observed at 14.6 ppm, whereas the signals for C3 and C15 were superimposed at 25 ppm. The remaining methylene carbon atoms gave rise to a broad multicomponent peak at about 35 ppm. Upon melting of the acid, three observations can be made (Figure 8B). First, the bandwidth of the peaks was reduced as a result of the increased molecular motions in the liquid state. This width reduction was less pronounced for the terminal methyl carbon, at 15 ppm, since this group already experiences a rotation in the solid acid—see the ^{2}H NMR spectra of PA-d_{31} for another manifestation of this motion.[33,37] Second, the peak associated with the carboxylic carbon vanished, probably because of the inefficiency of the cross-polarization when the mobility of the headgroup increases. Finally, with the exception of the C2 atom, the melting of the acid was accompanied by upfield shifts of the peaks associated with the methylene and methyl groups of the acyl chain. This shift was probably caused, in part, by a γ-gauche effect, similar to that reported in polymers, liquid crystal-forming molecules with long alkyl chains, and phospholipids.[38−41] The spectra of PA recorded at pH 9.0,

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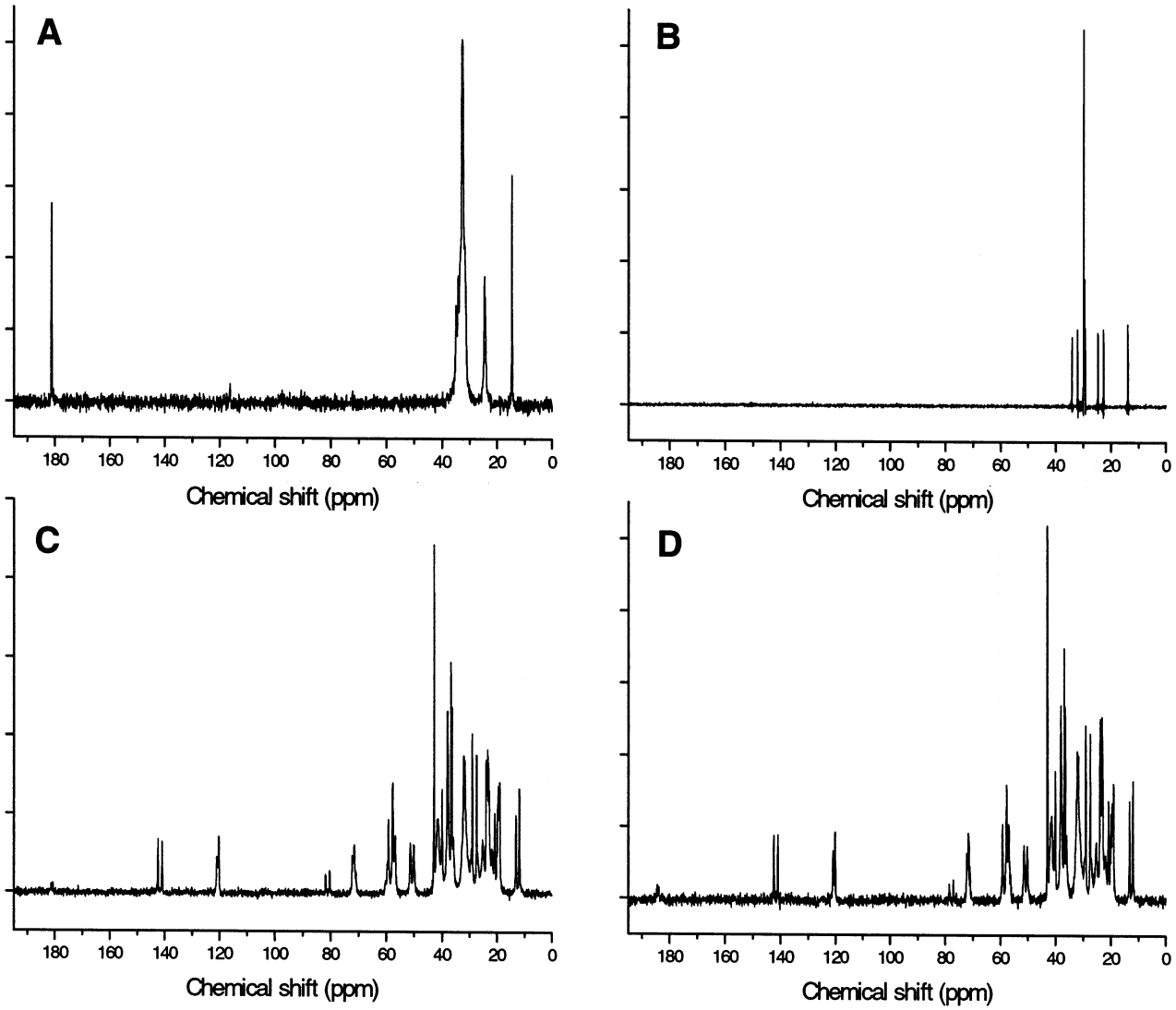

Figure 8. ^{13}C CP-MAS NMR spectra of hydrated PA and chol at pH 5.4: PA at (A) 25 and (B) 65 °C; cholesterol at (C) 25 and (D) 65 °C.

associated with the hydrated palmitate, showed peaks located in similar regions of the spectrum, but considerably broader, probably because of the heterogeneity of the sample in these conditions (data not shown).

^{13}C NMR spectra of cholesterol (Figure 8C,D) indicate that, in excess MES buffer and at temperatures below 70 °C, the sterol was in the monohydrate crystalline form. This was inferred from the observation of doublets for C5 (141 and 142 ppm), C9 (50 and 51 ppm), and C18 (12 and 13 ppm) and of a broad multiplet centered at 72 ppm for C3.[42] The presence of doublets has been explained by the presence of crystallographically nonequivalent molecules in the crystal lattice.[36] The presence of cholesterol as a crystalline monohydrate is also in agreement with our calorimetric data. No significant changes were observed between pH 5.4 and 9.

The ^{13}C NMR spectra of equimolar PA/chol mixtures at pH 5.4 are shown in Figure 9. At 25 °C, the spectrum resembled the summation of individual crystalline PA and cholesterol spectra (Figure 8A,C). This leads to the conclusion that, at 25 °C, both components are in the solid form, mostly as pure palmitic acid crystals and pure monohydrate cholesterol crystals. Additional NMR experiments with PA/chol mixtures, for which the cholesterol proportion varied between 10 and 85 mol %, provided similar results except that the intensity of the PA peaks relative to ones from cholesterol varied with the relative amount of each component (data not shown).

When an equimolar mixture at low pH was heated to over 50 °C, the spectrum changed radically (Figure 9B). First, the carboxyl carbon peak at 181 ppm disappeared, as in the case of molten pure acid. Second, the CH_3 peak of the palmitic acid shifted upfield from 15 ppm at 25 °C to 14 ppm. Its relative intensity was also decreased due to higher molecular mobility.[43] PA seemed to undergo a transition from a crystalline to a more disordered state, if one assumes that the shift of the CH_3 signal was due to the γ-gauche effect, and the disappearance of the carboxylic carbon was associated with increased motion. Cholesterol peaks indicated that the sterol was also involved in the transition. The C5, C6, C9, and C18 signals,

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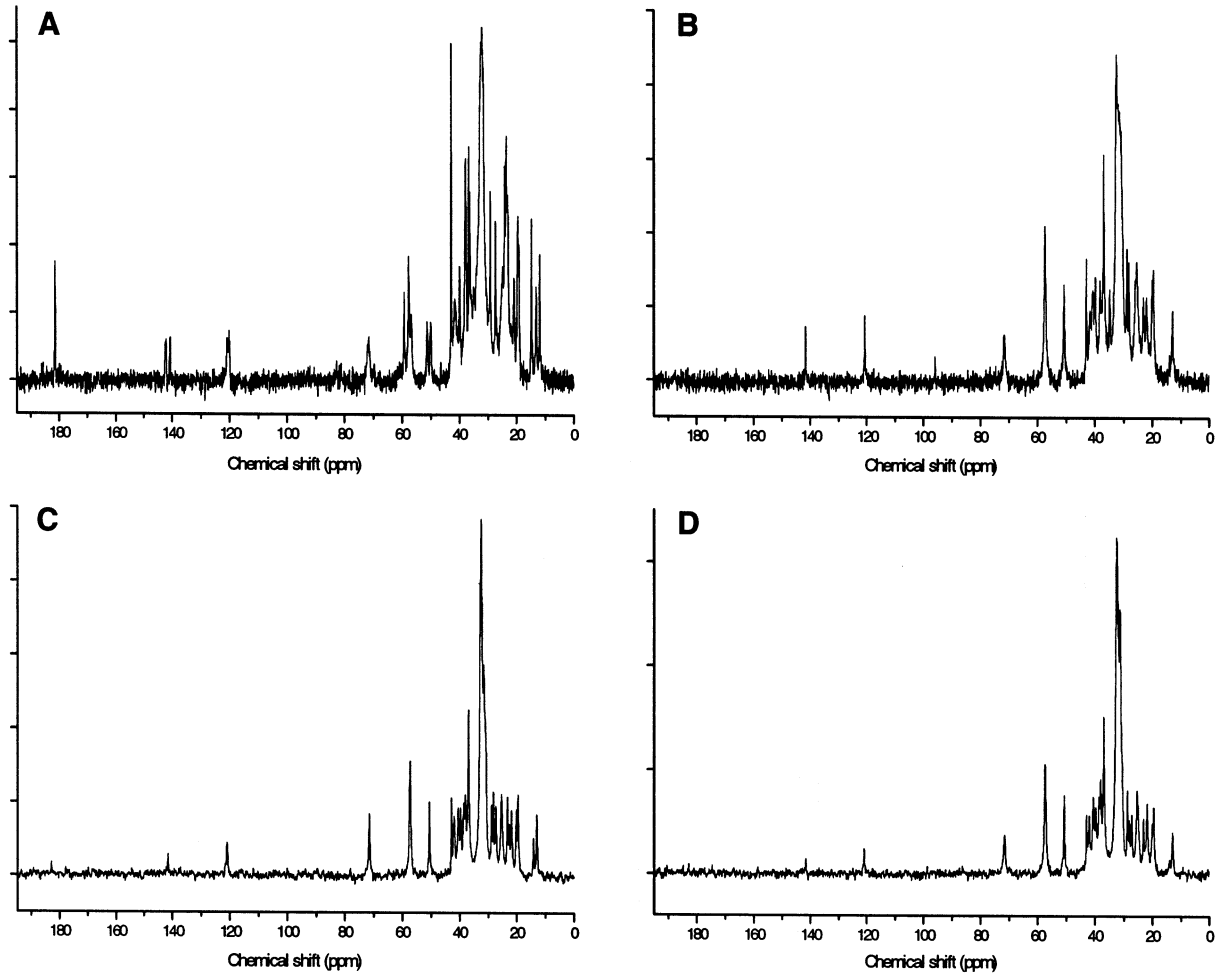

Figure 9. ^{13}C CP-MAS NMR spectra of 50/50 PA/chol mixture: pH 5.4 ((A) 25 and (B) 60 °C) and pH 9.0 ((C) 25 and (D) 60 °C).

Table 1. Lattice Spacings Obtained by X-ray Diffraction for the PA/chol System, as a Function of Temperature[a]

	lattice spacing, Å			
	25 °C	45 °C	55 °C	70 °C
50/50 PA/chol, pH 8.5	48.9 (lo phase)	49.2 (lo phase)	49.0 (lo phase)	49.5 (lo phase)
25/75 PA/chol, pH 8.5	34.6 (crystalline chol)	34.6 (crystalline chol)	34.6 (crystalline chol)	34.6 (crystalline chol)
	49.1 (lo phase)	49.1 (lo phase)	49.1 (lo phase)	49.1 (lo phase)
		22.4 (faint, unidentified)	22.4 (faint, unidentified)	22.4 (faint, unidentified)

[a] Structural attribution of each reported *d* spacing is in parentheses.

which were doublets for the crystalline form of cholesterol, became singlets located at 142, 121, 51, and 13 ppm, respectively. This phenomenon has previously been observed when cholesterol is solubilized in dipalmitoylphosphatidylcholine[41,44] and cholesteryl ester[36] matrixes.

The ^{13}C NMR spectra obtained at pH 9 showed significant differences relative to those at low pH. First, the spectrum of the PA/chol equimolar mixture at 25 °C (Figure 9C) showed singlets for C5 (142 ppm), C6 (121 ppm), C3 (71 ppm), C14 and C17 (57 ppm), C9 (50 ppm), and C18 (13 ppm) carbon nuclei of cholesterol. These features indicate that cholesterol was no longer in the crystalline monohydrate form and are consistent with cholesterol solubilized in the palmitate matrix. For cholesterol-rich mixtures (34/66 PA/chol), the C5 peak at 25 °C was a combination of the singlet peak characteristic of solubilized cholesterol and a multiplet previously observed for crystalline cholesterol (data not shown). However, the crystalline component vanished upon heating to 50 °C, where only a singlet was observed. In the case of palmitic acid signals, the CH_3 peak appeared at 14 ppm, a value near that obtained for the molten acid, and for PA in the lo phase obtained at low pH and high temperature. The temperature increase from 25 to 60 °C (Figure 9D) did not modify considerably the ^{13}C NMR spectrum.

X-ray Diffraction. XRD provided some information with regard to the types of phases present and their respective dimensions for PA/chol mixtures at pH 8.5. The main spacings derived from the XRD data are presented in Table 1.

For the equimolar mixture at pH 8.5, there was a single nontextured diffraction ring corresponding to a *d* spacing of 49 Å, observed between 25 and 70 °C. This observation is consistent with the existence of an lo phase. This spacing is larger than the *d* spacing of 39 Å measured for the dominant phase at temperatures higher than 55 °C for the equimolar PA/chol samples at pH 5.5. From the XRD data, no phase transition was observed, in agreement with

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the NMR and DSC observations. The results for the 25/75 PA/chol mixture, at pH 8.5, were similar, except that, over the whole investigated temperature range, an additional textured ring, corresponding to a *d* spacing of 34.6 Å, was observed and was attributed to the presence of crystalline cholesterol.

Discussion

A temperature−composition diagram for the PA/chol system at pH 5.5 was previously proposed.[11] It was indicated that, below 50 °C, both components of the PA/chol mixture are phase separated and in crystalline forms. The results presented here support these conclusions. The position of the ν_{CD} band in the IR spectra for an equimolar PA-d_{31}/chol mixture is typical of acyl chains with high conformational order,[11,28,45] and the splitting observed for the CD_2 deformation band indicates that the PA molecules form crystalline structures with an orthorhombic chain packing.[30,31,45] Since this splitting is associated with an intermolecular vibrational coupling, its presence indicates the formation of pure (or almost pure) PA-d_{31} domains that include a minimum of 100 molecules.[30−32,45] The results presented here provide additional details about the behavior of this system. Solid-state ^{13}C NMR observations also indicate that both PA and cholesterol are solid, since the sums of spectra associated with the individual crystalline lipids give the observed spectra for the PA/chol mixtures. In addition, we can specify that cholesterol exists under the monohydrate crystalline form. The presence of monohydrate cholesterol is reinforced by the lack of transition in the DSC trace around 33 °C, attributed to a polymorphic transition of anhydrous crystalline cholesterol.[24] All these observations show that PA and cholesterol seem to be immiscible under these conditions, in agreement with similar systems examined as Langmuir monolayers.[7,10]

When PA/chol mixtures at pH 5.5 are heated, the system undergoes between 50 and 55 °C a transition from crystalline to liquid ordered phase that involves both components. This transition is observed from the frequency shift of the C−D symmetric stretching, the merging of the two components of the CD_2 deformation, and the ^2H NMR spectra.[11] The DSC results presented here are consistent with this conclusion. An endothermic peak is observed around 53 °C (Figure 1). It was associated with the melting of an eutectic mixture with a composition that includes between 50 and 70 mol % cholesterol, as reported previously.[11,22] The other exothermic contribution in the thermograms, appearing at about 58.5 °C, is associated with the melting of the excess PA, when the fatty acid proportion is higher that that of the eutectic. The lamellar phase is also confirmed by the presence of a new prevailing diffraction ring at 55 and 70 °C, with a *d* spacing of 39 Å. The ^{13}C NMR spectra confirm the disordering of palmitic acid (Figure 9). The terminal methyl carbon and carboxylic acid carbon appear to be more mobile than in the crystalline state, as expected for a solid-to-fluid transition. The change in chemical shift for the methyl carbon is probably due to the presence of an increased amount of gauche conformation at the end of the acyl chain. This effect is also expected from the methylene carbons, but the observation of this spectral change is complicated by the overlap of this band with some other bands arising from cholesterol in the 20−40 ppm region. The ^{13}C NMR results reveal that cholesterol undergoes a parallel transition from the monohydrated crystalline form to a fluid one, since all the cholesterol signal characteristics of the solid state vanished with increasing temperature. There are no multiplet peaks for crystallographically nonequivalent carbon atoms, consistent with the fact that cholesterol molecules are solubilized into a lo phase involving PA. The absence of multiplets was previously observed when cholesterol was solubilized in cholesteryl ester or in dipalmitoylphosphocholine.[36,41]

The titration of the fatty acid in the equimolar PA-d_{31}/chol mixture was performed by infrared spectroscopy. The variations of the band areas associated with the carboxylate and carboxylic CO stretching modes as a function of pH indicate that the pK_a^{app} associated with PA in the mixture, at 25 °C, is around 8.2. This value is supported by the fact that the two components of the CD_2 deformation mode, observed at low pH, merge into a single component between pH 7.5 and 8. Therefore, it is assumed that the differences in the PA/chol binary system observed between pH 5 and 9 are mainly associated with the change of the protonation state of the fatty acid. This pK_a^{app} value is higher than those reported for fatty acid in the monomeric form, estimated to be about 4.8.[46,47] The pK_a^{app} value obtained for the PA/chol system is slightly higher than the values previously reported for long fatty acids in crystalline systems ($pK_a^{app} \sim 7.0$)[26] and similar to those reported for fatty acids incorporated in phospholipid matrix.[48−50] It is established that carboxylic groups at an interface display a pK_a^{app} shifted toward higher values than the free form as the negative interfacial charge density developed upon deprotonation creates a proton gradient between the interface and the bulk.[19,51] In addition, local interactions involving dielectric constant discontinuities have also been proposed as playing a role in such a shift.[19]

For values above pH 8.5, the PA/chol system does not experience any transition over the investigated temperature range (20−70 °C), as observed by DSC, IR, and ^2H NMR spectroscopies. All observations presented here converge to the conclusion that a thermally stable lo phase prevails at high pH. The ^2H NMR spectra of PA-d_{31} in the mixtures with cholesterol at high pH are typical of a lo phase. The order profiles derived from these spectra show that the region of the acyl chain near the polar headgroup (the plateau region) is highly ordered, with order parameters slightly larger than 0.4, similar to those observed for phospholipids in the lo phase (e.g. see refs 2, 3, and 52). In IR spectroscopy, the symmetric ν_{CD} mode displays a relatively constant position at 2091 cm^{-1}, over the investigated temperature range. This value is similar to those previously observed for phospholipid matrixes in the lo phase (e.g. see refs 3, 11, and 29). In the region of the CD_2 deformation mode, no splitting indicative of crystalline phase with orthorhombic symmetry was observed. X-ray diffraction data also support the formation of a dominant lo phase. Lamellar spacings of 49 Å (Table 1) are observed, a value about 10 Å greater than the lo phase formed at low pH. Because the hydrophobic thickness of the bilayers seems to be rather insensitive to pH, as inferred from the similar orientational order profiles,

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this difference is associated with thicker water layers between lipid bilayers. This increased hydration is most likely attributed to the charged interface created by the carboxylate groups. Cholesterol is solubilized in the palmitate matrix at high pH values. As in the case of the equimolar mixture at low pH and $T \geq 60$ °C, the signals of C5, C9, and C18 in the ^{13}C NMR spectra of the mixtures at high pH are singlets, meaning that cholesterol is no longer in the crystalline monohydrate form.

For equimolar PA/chol ratios, the lo phase seems to be the unique lamellar phase in the system, since only one reflection is detected by X-ray diffraction. All the cholesterol is solubilized in the lo phase, as inferred from the absence of a transition associated with crystalline cholesterol as observed by calorimetry and ^{13}C NMR spectra. When the cholesterol molar ratio is raised to 75 mol %, excess cholesterol crystallizes, indicating that the solubility limit of cholesterol in the palmitate matrix is reached. This solid form of cholesterol gives rise to the typical *d* spacing of 34.6 Å observed from an X-ray diffraction textured ring. Moreover, there is a coexistence of the C5 signals for the crystalline monohydrate and the solubilized forms of cholesterol, in the ^{13}C NMR spectra (data not shown). Our findings on palmitate/chol systems indicate that very few PA molecules are required to solvate cholesterol (1 to 1) and to lead to the formation of stable lo lamellar phases.

Above its melting temperature, the pure fully deprotonated PA forms micellar aggregates.[26] The present results show that the presence of cholesterol in the macroassembly leads to stable bilayers. Because of its ordering capacity, cholesterol induces the formation of bilayers where the acyl chains are fluid but ordered. For the protonated PA, this behavior is only observed upon melting.[11] Consequently, it is proposed that increased loosening of the PA molecules packing, as a result of increased temperature at low pH, and the electrostatic repulsion of carboxylate at high pH, promotes the insertion of cholesterol that, as a result, stabilizes the lamellar structure. It was shown that vesicles can be prepared from unprotonated saturated fatty acids and fatty alcohol.[25] It was proposed that the creation of a hydrogen bond network was required for the stability of the bilayers prepared with monoacyl chain surfactants. The present results are consistent with this hypothesis because the hydroxyl group of cholesterol can participate in similar hydrogen bonding with the palmitate carboxylate. However, a distinctive aspect of these bilayers is that the acyl chains of PA are highly ordered. The formation of an lo phase in mixtures with unprotonated fatty acids and cholesterol strengthens the idea that the induction of an lo phase by cholesterol is a general feature.

In conclusion, it is interesting to note that many years ago Ekwall and collaborators, on the basis of observations made with optical microscopy, reported that soaps of fatty acids were able to solubilize crystalline cholesterol and induce the formation of mesomorphous forms, a phenomenon that was favored as the hydrocarbon chain of the fatty acid soap becomes longer.[53] It is likely that the resulting liquid crystalline form included at least partly some lamellar lo phase. The formation of a lamellar phase by single-chain amphiphiles such as PA, in the presence of cholesterol, supports the conclusion of Hargreaves et al. indicating that liposomes can be formed from ionic soaps in the presence of other uncharged amphiphile molecules.[25] The unprotonated PA/chol system leads to a stable lo lamellar phase, and its formation is associated with the following requisites: (a) inter-headgroup electrostatic repulsion favoring cholesterol intercalation; (b) the ability of cholesterol to both disrupt lipid packing and order acyl chains; (c) the possibility of establishing a hydrogen-bond network involving both cholesterol and PA at the lipid/water interface. We are currently examining the impact of each factor to propose, in future, reliable requirements for the development of simple nonphospholipid lo-phase liposomes.

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